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THIDIAZURON-INDUCED HIGHLY MORPHOGENIC CALLUS AND HIGH FREQUENCY REGENERATION OF FERTILE PEANUT (*ARACHIS HYPOGAEA* L.) PLANTS

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SUMMARY

An efficient regeneration system was developed by culturing immature cotyledons and embryo axes of *Arachis hypogaea* L. cv. Georgia Green on Murashige and Skoog basal medium (MS) supplemented with various concentrations of thidiazuron (TDZ; 1, 5, 10, and 15 μ*M*). Highly morphogenic callus was produced from 100% of the explants comprising the cotyledon with attached embryo axis when cultured in the dark on 10 μ*M* TDZ. Upon excision and continued culture in the dark on 10 μ*M* TDZ, morphogenic callus grew repetitively during monthly subcultures and retained its regeneration potential. For organogenesis, a gradual reduction in TDZ concentration and exposure to light were necessary before transfer to MS basal medium. Inclusion of indole-3-butyric acid in liquid MS medium favored rooting of recovered shoots. A distinct feature of this investigation is the induction of highly morphogenic callus by TDZ and regeneration of morphologically normal, fertile peanut plants after 8 months of callus subculture.

Key words: repetitive growth; groundnut.

INTRODUCTION

Arachis hypogaea L. is a major protein-rich grain legume cultivated worldwide primarily for human consumption. Recently, a genetic engineering approach is being applied as an adjunct to conventional breeding for improvement of nutritional quality and agronomic attributes of peanuts (Knauft and Ozias-Akins, 1996). Successful transformation of tissue by *Agrobacterium tumefaciens* or microprojectile bombardment followed by recovery of transgenic plants usually requires an efficient in vitro regeneration system. Development of transgenic plants through somatic embryos thus far remains the pathway of choice due to their single-cell origin and conversion ability to genetically stable, nonchimeric plants. Embryogenic cultures can be established with many genotypes of peanut from either immature (Hazra et al., 1989; Ozias-Akins, 1989; Sellars et al., 1990) or mature seed explants (McKently, 1991; Gill and Saxena, 1992; Saxena et al., 1992; Baker et al., 1995). The main disadvantages of immature seed explants are their seasonable availability and the difficulty of determining an exact developmental stage from external pod morphology. These variations can be minimized by use of readily available dry mature seeds as an initial source material (Baker et al., 1995; McKently, 1995). However, mature peanut seeds are not clean enough to yield aseptic in vitro cultures due to the underground origin of pods, which often do not respond uniformly. These problems can be avoided by establishing repetitively growing morphogenic cultures which are an ideal source of tissue for application of gene transfer techniques. Repetitively growing cultures ensure greater tissue homogeneity than most primary explants which is a distinct advantage for elevating transformation frequency. Furthermore, the gradual proliferation of transformed sectors due to the expression of a selectable marker gene and the elimination of nontransformed tissue during prolonged selection reduces the possibility of recovering chimeric plants.

A variety of growth regulators can induce somatic embryogenesis or organogenesis in peanut seed explants including different auxins (Hazra et al., 1989; Ozias-Akins, 1989; Baker and Wetzstein, 1994; McKently, 1995) and the substituted phenylurea, thidiazuron (TDZ) (Gill and Saxena, 1992; Saxena et al., 1992; Kanyand et al., 1994; Li et al., 1994). Thus far, repetitively growing embryogenic cultures of peanut can be reproducibly initiated from multiple genotypes by use of picloram (Ozias-Akins et al., 1992a) or from a limited number of genotypes with 2,4-dichlorophenoxyacetic acid (2,4-D) (Durham and Parrott, 1992). Repetitively embryogenic cultures on picloram were used to produce the first transgenic peanut plants (Ozias-Akins et al., 1993). In this communication, we report an alternate regeneration system for peanut based on the establishment of repetitively growing, highly morphogenic cultures induced by TDZ that remained morphogenic for 8–9 months and differentiated morphologically normal fertile plants.

MATERIALS AND METHODS

Plant material and culture initiation. Immature pods and seeds of *Arachis hypogaea* L. cv. Georgia Green obtained from field-grown plants 3–4 wk after pegging were surface sterilized essentially according to Ozias-Akins et al. (1992b). Seeds were opened aseptically under a dissecting microscope to separate the cotyledons after the seed coats were removed. Cotyledons with attached embryonic axes as well as separated cotyledons and embryo axes were cultured in plastic petri dishes (100 × 15 mm, Fisher Scientific Co., Pittsburgh, PA) containing 20 ml culture medium that consisted of Murashige and Skoog (1962) salts and vitamins plus 3% sucrose (MS) containing 0.8% agar and 1, 5, 10, or 15 μ*M* TDZ (*N*-phenyl-*N*[1]-1,2,3-thiadiazol-5-yl urea; a gift from Nor-Am Chemical Co., Wilmington, DE). All media were adjusted

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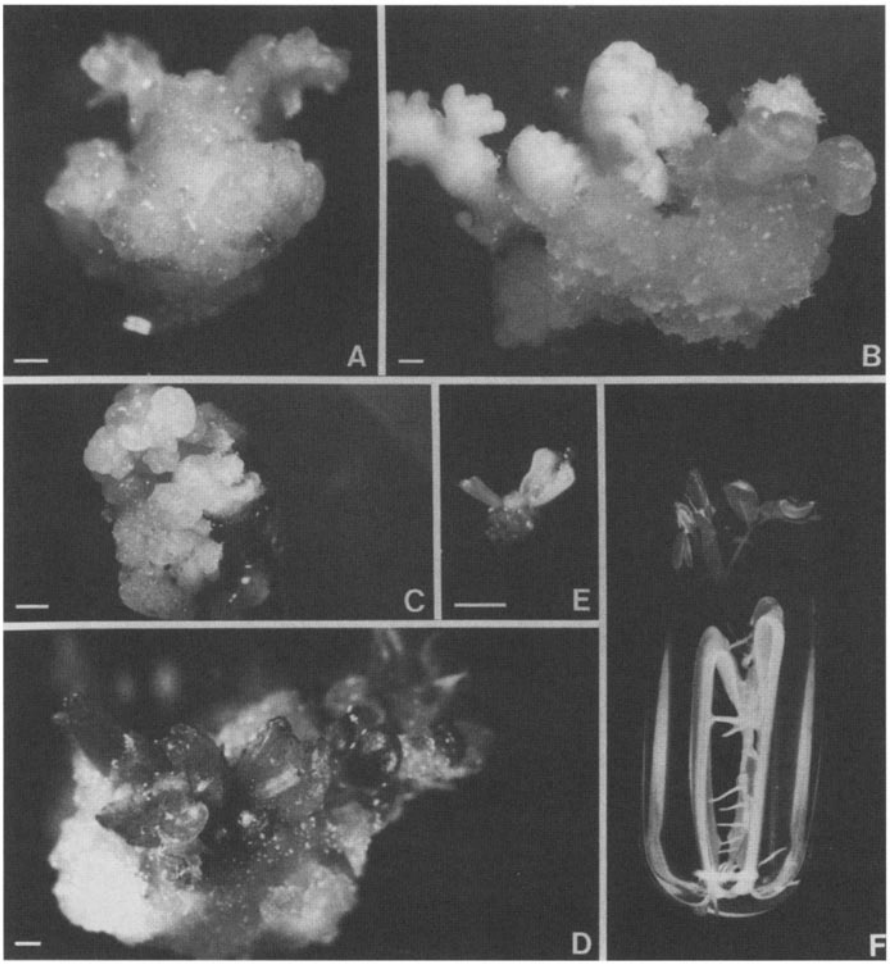

FIG. 1. *A*, Induction of highly morphogenic callus from the epicotyledonary region of an *Arachis hypogaea* embryo axis with cotyledon attached (cotyledon was removed for photography) on 10 μ*M* TDZ. *B*, Differentiation of highly morphogenic callus into translucent or opaque types and nonmorphogenic callus after 4 wk on maintenance medium. *C*, Highly morphogenic, opaque callus during monthly subcultures on maintenance medium. *D–E*, Redifferentiation of highly morphogenic callus into shoot buds (D) and structures resembling somatic embryos (E) after transfer to MS + 5 μ*M* TDZ. *F*, Development of roots after shoots were transferred to MS + IBA (4.92 μ*M*) for 4 wk on paper rafts. Bars in *A–E* equal 1 mm.

to a pH of 5.8 and sterilized by autoclaving at 0.122 MPa for 20 min. Cultures were incubated in the dark at 28° C for initiation as well as maintenance of highly morphogenic callus. After 4 wk, the number of explants from each treatment producing morphogenic callus was recorded. For statistical analysis, a split-plot design was used with explant as the main plot and concentrations as subplots. Proc MIXED (SAS, 1992) was used to analyze the untransformed data.

Maintenance of morphogenic cultures. After 4–6 wk, morphogenic calluses that originated from the cotyledon-attached embryo axes were separated from the regions surrounding the apex of zygotic embryos (epicotyledonary region) with a sharp scalpel under a stereomicroscope and subcultured on the same medium. Subcultures were maintained in the dark on the same TDZ concentration used for initiation, and the maintenance medium additionally contained 1 g each filter-sterilized glutamine and asparagine per l. Long-term maintenance of morphogenic callus took place by regular subcultures at monthly intervals.

Regeneration of plants. For plant regeneration, morphogenic calluses originated and maintained on maintenance medium with 10 μ*M* TDZ were first transferred to 5 μ*M* TDZ for 1 month under light. Following this stepwise reduction in TDZ concentration, highly morphogenic calluses were transferred either directly to MS medium with activated charcoal (0.25%) for organogenesis or to one of three intermediate media (MS + 1.44 μ*M* gibberellic acid (GA_3); MS + 2.32 μ*M* kinetin (KIN) + 1.44 μ*M* GA_3; MS + 22.2 μ*M* 6-benzylaminopurine (BA) + 1.0 μ*M* TDZ) for 1 mo. before transfer to MS medium with activated charcoal (0.25%). Shoots regenerated on the GA_3-KIN combination were used for a rooting experiment and were transferred once again to charcoal-supplemented medium (0.25%) before rooting. For rooting, shoots (20 per treatment) of uniform length were placed on paper rafts (Whatman 3 MM Chromatography Paper) immersed in liquid MS medium supplemented with various concentrations (0, 4.92, 7.38, or 9.85 μ*M*) of indole-3-butyric acid (IBA). More than 80 plants with fully developed root systems were washed several times with tap water before being transplanted to sterile Fafard® no. 2 potting mix (Conrad Fafard, Inc. Agawam, MA). For all steps of regeneration, cultures were maintained under light (an average of 100 μmol m^{-2} s^{-1} from cool-white fluorescent lamps for a 16-h light/8-h dark photoperiod) at 28° C.

RESULTS

Induction of morphogenic callus. Irrespective of TDZ concentration in the nutrient medium, all three explant types enlarged within a month in the dark and retained their original cream color. The only response of the explants cultured on MS basal medium was occasional germination of embryo axes to produce young seedlings. The shoot tip of embryo axes, both attached to and detached from cotyledons, ceased to elongate in TDZ-supplemented medium, and the first leaf pair thickened. After 4–6 wk, nodular, actively proliferating, cream-colored callus developed from tissue subtending the apex of the embryo axis near the cotyledonary nodal region (Fig. 1 *A*). This type of nodular callus was highly morphogenic and usually was accompanied by a friable nonmorphogenic callus in the first few subcultures (Fig. 1 *B*). In cotyledon explants, morphogenic callus occasionally originated at the cotyledonary notch, near the site of detachment of the embryo axis, and rarely toward the peripheral

TABLE 1

FREQUENCY OF INDUCTION OF MORPHOGENIC CALLUS FROM SEED EXPLANTS OF *ARACHIS HYPOCAEA* CULTURED ON MS MEDIUM SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF TDZ[a]

Explant[b]	Thidiazuron concentration (μM)[b] 1.0	5.0	10	15
Cotyledon with attached embryo axis	91.7%	95.0%	100%	100%
Cotyledon	5.0%	8.3%	18.3%	11.7%
Embryo axis	11.7%	16.7%	35.0%	31.7%

[a]Thirty explants of each type were cultured to determine callus initiation response. Data were collected after 4 wk; values given are an average of two experiments.

[b]SE = 1.29; F(explant)(2,3) = 2852**; F(conc)(4,12) = 847**; F(int)(8,12) = 298**.

TABLE 2

REGENERATION RESPONSE OF MORPHOGENIC CALLUS OF *ARACHIS HYPOCAEA* ON TRANSFER TO MS BASAL MEDIUM WITH OR WITHOUT AN INTERMEDIATE SECONDARY MEDIUM[a]

Growth regulators in secondary medium	Percentage of calluses developing shoots	Average number of 3–4-cm-long shoots per callus ± SE
None	100	1.71 ± 0.05
GA_3 (1.44 μM)	100	2.27 ± 0.03
GA_3 (1.44 μM) + KIN (2.32 μM)	100	3.71 ± 0.15
BA (22.2 μM) + TDZ (1.0 μM)	100	1.24 ± 0.05

[a]Note that 100 opaque calluses (original inoculum approximately 5-mm callus pieces) that originated on MS + 10 μM TDZ and subsequently were transferred to MS + 5 μM TDZ were used for each secondary medium. Data were collected after 4 wk. Values given are an average of two experiments ± SE (100 calluses per experiment).

margin of the cotyledons. Embryo axes attached to cotyledons were the most responsive explant for the induction of morphogenic callus across all TDZ concentrations (Table 1). The lower concentrations of TDZ (1 and 5 μM) induced morphogenic callus which remained embedded in the surrounding nonmorphogenic callus. The percentage of explants that produced morphogenic callus was greatest at 10 μM TDZ for all explant types (Table 1). The highest concentration of TDZ tested (15 μM) also induced morphogenic callus although it caused excessive blackening of the explants.

Influence of TDZ concentration on maintenance of morphogenic callus. Upon separation of morphogenic callus from the explant and subculture on TDZ-supplemented medium, 50–100% of primary calluses underwent repeated multiplication. All calluses on 10 μM TDZ continued to proliferate, whereas less than 80% of calluses on media with other TDZ concentrations proliferated. Therefore, subsequent growth of the primary callus largely was a function of the TDZ concentration in the induction and maintenance medium. Morphogenic calluses initiated, maintained, and subsequently transferred to MS medium with 1 μM TDZ were overwhelmed by the growth of surrounding nonmorphogenic callus and ceased repetitive growth within 2–3 wk. Similarly, the growth of morphogenic callus initiated on 5 μM TDZ could not be sustained during subsequent subcultures on medium with 5 μM TDZ. Morphogenic calluses induced in 10 μM TDZ were morphologically similar to those raised in 1 or 5 μM TDZ-supplemented medium, and continuously proliferated when maintained on this concentration of TDZ. The highest concentration of TDZ (15 μM) caused primary morphogenic calluses to turn brown and inhibited growth. Since TDZ at 10 μM was optimum for the induction and proliferation of morphogenic callus, it subsequently was used for regular maintenance.

Morphology of morphogenic calluses. Two to three weeks after the first subculture, callus induced on 10 μM TDZ produced two types of cream-colored secondary morphogenic calluses, one opaque-compact and another translucent-friable, in addition to nonmorphogenic callus (Fig. 1 *B*). In subsequent subcultures the growth of nonmorphogenic callus gradually subsided (Fig. 1 *C*). Small clumps of both types of morphogenic callus could be carefully separated and further multiplied as two types of morphogenic cultures. Both types of callus exhibited a uniform repetitive growth for 8–9 months without losing their ability to differentiate. The opaque callus was slow-growing, compact, and highly morphogenic compared to the translucent callus; thus, the former was used for redifferentiating plants.

Organogenesis in morphogenic callus. After induction, a primary requirement for redifferentiation of morphogenic callus was a gradual reduction of TDZ in the medium. Direct transfer of morphogenic callus to MS basal medium promoted prolific growth of nonmorphogenic callus within a month, effectively preventing redifferentiation. We transferred 100 callus pieces of both types of callus (i.e., translucent or opaque originated on 10 μM TDZ) to 5 μM TDZ instead of direct transfer to basal MS. Within a month of transfer to light, both opaque and translucent callus turned green and showed signs of differentiation (frequencies of 100% and 64%, respectively). Calluses predominantly differentiated shoots (Fig. 1 *D*), though infrequently, structures morphologically similar to bipolar somatic embryos also were observed (Fig. 1 *E*). In subsequent subcultures, these embryo-like structures did not develop a distinct root while development of the shoot progressed normally. Gradually, a clear distinction was lost between shoots produced through organogenesis or from these embryo-like structures.

Influence of various growth regulators on shoot elongation. As described in the previous section, shoots routinely were regenerated from long-term morphogenic calluses induced on 10 μM TDZ upon transfer to 5 μM TDZ followed by basal medium. Shoots that developed on TDZ medium generally were clustered and exhibited slow elongation of shoot buds (Fig. 1 *D*). This influence of TDZ could be counteracted by transferring shoots from primary medium causing maximum shoot proliferation (MS + 5 μM TDZ) to secondary media supplemented with different plant growth regulators with or without TDZ for elongation of shoots (Table 2). An obvious elongation response (3–4 cm) was observed on media containing 1.44 μM GA_3. The largest number of elongated shoots was found on medium containing 1.44 μM GA_3 + 2.32 μM KIN. Transfer of clustered shoot primordia for a second passage on basal MS medium facilitated recovery of an additional five or six elongated shoots per callus piece. A combination of 22.2 μM BA + 1.0 μM TDZ caused excessive browning of callus with occasional elongation of shoot primordia.

For rooting, shoots that originated on MS + 1.44 μM GA_3 + 2.32 μM KIN were transferred to MS + 0.25% charcoal for a month and then to MS medium with or without IBA after basal callus was excised. Shoots continued to elongate within 3–4 wk regardless of IBA concentration. Callus continued to be produced at the base of the

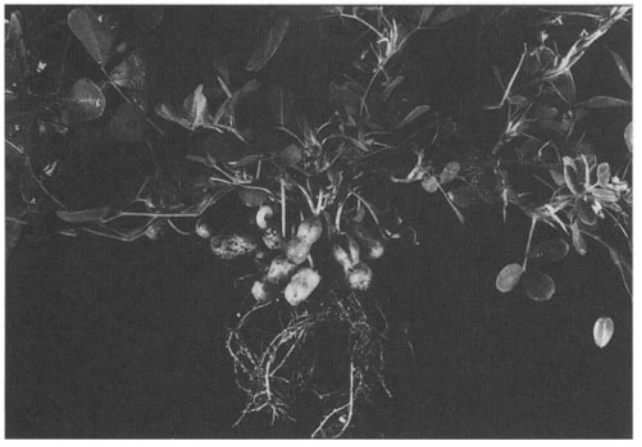

FIG. 2. Fertile *Arachis hypogaea* plant, 6 months after transplantation to soil.

stem in basal MS medium which hampered rooting (30% of shoots rooted). In contrast, IBA promoted a well-developed adventitious root system directly from the stem within a month of culture (Fig. 1 *F*). Roots were induced faster on 4.92 μ*M* IBA (75% of shoots rooted) than MS basal medium. Higher concentrations of IBA (7.38 or 9.85 μ*M*) induced callus at the cut surface of the shoots, and root development was inhibited. However, this callus could be excised, and the shoots readily produced roots after transfer to basal MS.

The plantlets originating from TDZ-induced morphogenic callus developed normally with no aberrant shoot morphology, stunting, hyperhydricity, or poor rooting ability. Approximately 86% (84/98) of the transplanted plants grew to maturity within 2 months, flowered, and developed gynophores that produced pods with seeds (Fig. 2). In comparison with seed-derived plants, relatively small TDZ-derived plants flowered rapidly and profusely. It is evident from the present investigation that TDZ did not exert any persistent adverse influence on rooting ability of shoots and recovery of morphologically normal plants from long-term TDZ-grown cultures.

DISCUSSION

Our experiments have explored the effects of thidiazuron on the dedifferentiation and redifferentiation ability of meristematic target tissues of *Arachis hypogaea* during multiple culture cycles. Thidiazuron is a substituted phenylurea which was first developed as a cotton defoliant (Arndt et al., 1976) and now is used as a potent growth regulator in diverse plant species for eliciting a wide spectrum of in vitro responses (for a recent review *see* Murthy et al., 1998). Thidiazuron most often exerts cytokinin-like activity and generally induces shoot buds from cultured tissues by causing an accumulation of cytokinins either due to rapid conversion of ribonucleotides to biologically active ribonucleosides (Capelle et al.,1983) or due to de novo synthesis of purine cytokinins (Thomas and Katterman, 1986). Thidiazuron also has been proposed to induce morphogenesis in vitro by evoking a stress response (Murthy et al., 1998). Alternatively TDZ could induce morphogenic callus through its elicitation of both auxin and cytokinin responses as shown in geranium (Gill et al., 1993) and tobacco (Gill and Saxena, 1993).

In the presence of TDZ, the structural integrity of the peanut cotyledon attached to the embryo axis emerged as a crucial factor for enhancing in vitro morphogenesis in the present investigation, probably due to its role as a nutrient and growth regulator reservoir (Bates et al., 1992; Saxena et al., 1992; Li et al., 1994; Murthy et al., 1995). Our results showed that a range of TDZ concentrations (1, 5, 10, or 15 μ*M*) effectively induced actively proliferating, highly morphogenic callus from epicotyls attached to cotyledons, but the optimum level for repetitive growth was 10 μ*M*. This optimum concentration of TDZ has been remarkably uniform in diverse crop species, namely, *Fraxinus americana* (Bates et al., 1992), *Glycine max* (Kaneda et al., 1997), *Pisum sativum* (Bohmer et al., 1995) and peanut (Gill and Saxena, 1992; Saxena et al., 1992; Li et al., 1994) with the exception of valencia-type peanuts, for which a high concentration of TDZ (136.36 μ*M*) was most effective (Kanyand et al., 1994). We observed that highly morphogenic callus remained prolific only at 10 μ*M* TDZ, whereas repeated cultures on lower concentrations triggered a shift to a nonmorphogenic state and a higher concentration caused excessive blackening of explants. Explants of peanut grown on TDZ medium previously have been shown to dedifferentiate into nonmorphogenic callus by lowering TDZ levels (Gill and Saxena, 1992; Kanyand et al., 1994). Apart from induction and maintenance, the redifferentiation process was also markedly influenced by the concentration of TDZ in the culture medium. Shoots were routinely regenerated by successive transfer of morphogenic calluses from 10 μ*M* TDZ to 5 μ*M* TDZ followed by basal medium.

Repetitively growing morphogenic cultures have been key to successful transformation in many agricultural crops due to their rapid proliferation, the ease and effectiveness of applying selection, and the ability to regenerate transformed plants. Transgenic plants of most of the recalcitrant crops (legumes and cereals, for example) have been produced by subjecting repetitively growing tissues to particle bombardment or cocultivation with *Agrobacterium* (Hinchee et al., 1994). Such repetitively growing, highly morphogenic cultures can be induced in vitro from certain cells of immature zygotic embryos, seedling tissues, or young inflorescences in response to phytohormones. Immature cotyledons, embryo axes, or young leaves of peanut routinely produce somatic embryos in response to the auxins, 2,4-D, naphthaleneacetic acid (NAA), or picloram (Hazra et al., 1989; Ozias-Akins, 1989; Baker and Wetzstein, 1994). The embryo axis of peanut has repeatedly been used to develop regenerable cultures due to the high density of meristematic cells (McKently, 1991; Saxena et al., 1992; Kanyand et al., 1994; Li et al., 1994). Our results demonstrate that the entire epicotyledonary region of the immature embryo axis can produce highly morphogenic callus in response to TDZ that remains morphogenic under long-term maintenance and efficiently regenerates morphologically normal peanut plants. In previous studies, peanut tissues cultured on TDZ-supplemented medium produced clearly differentiated somatic embryos (Gill and Saxena, 1992; Saxena et al., 1992) or shoot buds (Kanyand et al., 1994; Li et al., 1994) from the primary explant; however, we were able to induce highly morphogenic callus from similar explants on similar media. The less differentiated nature of our cultures may be due to the combination of explant source (immature rather than mature seeds) and continuous incubation of cultures in the dark (instead of light).

Shoot buds induced in response to TDZ generally are clustered and do not readily elongate. In some plant species, poor elongation of TDZ-induced shoots has been counteracted by transfer of shoots from the shoot proliferation medium to an elongation medium either lacking TDZ or supplemented with a different combination of plant

growth regulators (Huetteman and Preece, 1993). In the present report, we observed that a medium containing GA_3 was required for optimal elongation of shoots that had differentiated on MS + 5 μM TDZ. The addition of kinetin to the GA_3-containing medium further enhanced the number of elongated shoots. Livingstone and Birch (1995) observed substantial elongation of TDZ-derived shoots on 22.2 μM BA medium, although the same concentration of BA did not bring about noticeable improvement in elongation of TDZ-derived peanut shoot primordia in our experiments. However, due to the inclusion of TDZ (1 μM) in the BA (22.2 μM)-supplemented medium, our results are not directly comparable with the previous report.

Since TDZ is highly stable and resistant to degradation by cytokinin oxidase (Mok et al., 1987), its persistence generally hampers rooting in shoots induced in response to TDZ (Huetteman and Preece, 1993). These effects usually can be overcome by inclusion of an auxin in the rooting medium. For example, IBA facilitated rapid development of the root system from TDZ-induced shoots of several legumes whereas basal medium promoted the formation of callus at the base of shoots that hampered rooting (Kartha et al., 1981). Although we were able to efficiently root TDZ-induced peanut shoots on low concentrations of IBA, other treatments such as media containing charcoal (Kanyand et al., 1994) or NAA (Li et al., 1994) may be equally effective.

Previously, we have observed that plants regenerated from long-term cultures of picloram-induced transgenic somatic embryos exhibited delayed flowering and low fertility (Singsit et al., 1997). Although our present results show that long-term maintenance of highly morphogenic callus on TDZ did not adversely affect regeneration potential or plant growth, flowering, fertility, and seed development, the fertility of transgenic plants from long-term TDZ-derived, transformed cultures remains to be tested.

In conclusion, an alternative repetitive culture system consisting of highly morphogenic callus that can be maintained for extended periods and yet continue to differentiate large numbers of morphologically normal and fertile plants has been developed. The culture system described in the present investigation is not labor intensive, requires only simple maintenance by monthly subculture, and enables access to available tissue for transformation experiments.

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